Ca^{2+} loss from these intracellular stores, not from the extracellular space, we developed a flexible in vitro 3D skin equivalent model that allows intra-organelle monitoring of calcium levels by genetically encoded sensors. These new findings suggest that long-held hypotheses addressing Ca^{2+} control of differentiation and barrier formation require revision.

3124-Pos Board B279

Store-Operated Ca^{2+} Entry is a Tonic and Phasic Signal in Skeletal Muscle

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Store-operated Ca^{2+} entry (SOCE) is activated very rapidly in skeletal muscle upon depletion of $[Ca^{2+}]_{SR}$ below an activation threshold due to a prepositioning of Orai1 and STIM1L uniformly throughout the junctional membranes (Edwards et al 2010, Cell Calcium, Darbellay et al, 2011, J Cell Biol). Its physiological role appears to be signalling, not refilling the sarcoplasmic reticulum (SR) (Launikonis et al 2010, Pflügers Arch). If this is the case then the rate, amplitude and frequency of SOCE during the release of SR Ca^{2+} is important. To examine SOCE kinetics during SR Ca^{2+} release we imaged cytoplasmic rhod-2 with fluo-5N inside t-system or SR of skinned fibres from mouse fast-twitch muscle, to measure Ca^{2+} release with $[Ca^{2+}]_{t\text{-}sys}$ or $[Ca^{2+}]_{SR}$, respectively. Ca^{2+} release was induced by lowering $[Mg^{2+}]_{cyto}$, causing a slow Ca^{2+} release. Step changes in $-d[Ca^{2+}]_{SR}/dt$ and $-d[Ca^{2+}]_{SR}/dt$ during cell-wide Ca^{2+} transients and waves were observed. Imaging of SR fluo-5N indicated three states of SR Ca^{2+}-buffering, dropping in a stepwise manner because the SR Ca^{2+}-buffer calsequestrin (CSQ) reduces its aggregation state and affinity for Ca^{2+} with the progressively lowered total SR calcium, allowing $[Ca^{2+}]_{SR}$ to change more freely with each step (Launikonis et al 2006, PNAS). SOCE was active in the phases of reduced CSQ aggregation. SOCE was active in the two latter phases and its rate was always proportional to $-d[Ca^{2+}]_{SR}/dt$. There was a tonic response during the cell-wide transient, then a fast, phasic SOCE response during repetitive Ca^{2+} waves. Phasic SOCE signal amplitude could vary by a factor of 10 but was always proportional to depletion of $[Ca^{2+}]_{SR}$. Our results show that the kinetics of SOCE signals are tightly regulated by $[Ca^{2+}]_{SR}$ and we predict that these signals can be decoded by the muscle for gene expression.

3125-Pos Board B280

A Method to Characterize Calcium Activity in Stimulated Cultures of Cardiac Myocytes

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Cardiac function and disease constitutes a complex physiological process involving several biophysical scales, from the stochastic dynamics of a single calcium release channel to the calcium signal of a single myocyte and the activation of contraction across a cardiac tissue. Integration of signals from several of these scales requires specific tools to study their interdependence.

We present a method for automatic detection of calcium signals in cultured cardiomyocytes subjected to external field stimulation. The method is applied to a sequence of confocal fluorescence images, and provides information on both the calcium activity in individual myocytes, the global calcium signal of the imaged field, as well as the propagation of the calcium signal across the cell culture.

The approach first segments each cell in the culture and computes its average calcium activity. An automatic classification method then identifies the response of each cell among six different dynamical regimes a) uniform response, b) alternating response, c) irregular response, d) calcium waves, e) phase-lock (conduction block) or f) inactive. The system computes the area, the full duration at half maximum (FDHM), the resting value and the peak of the calcium transient of each cell. Subsequently, it maps the distribution of cells from each group within the imaged field.

Finally, the method generates an isochronal map of the activation of each cell in the culture as the calcium front propagates across the culture, computing the linear and angular velocities as well as the propagation direction.

The resulting data can be used to quantify how abnormal calcium regulation at the single-cell level affects the propagation of the calcium signal in a myocyte culture. The method is also suitable for a quantitative comparison of the effects of pharmacological or genetic treatments of cell cultures.

3126-Pos Board B281

Improved Protocol to Record Sarcoplasmic Reticulum Calcium Concentrations in Mouse Cardiomyocytes

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In cardiomyocytes, the sarcoplasmic reticulum (SR) plays a very important role as the Ca^{2+} store. During excitation-contraction coupling Ca^{2+} is released from the SR through ryanodine receptors (RyRs) to the cytosol to activate the contraction. Deregulations in the SR-Ca^{2+} release mechanism can be associated with cadiopathologies (e.g. arrhythmias, CPVT, heart failure). Alterations of the SR Ca^{2+} concentration ($[Ca^{2+}]_{SR}$) can be explained by dysfunctions of elements that participate in the Ca^{2+} uptake/release balance (SERCA and RyR). Recently, the interest to measure $[Ca^{2+}]_{SR}$ directly has led to the application of low affinity Ca^{2+} indicators (mag-fluo-4, fluo-5N) to quantify changes of $[Ca^{2+}]_{SR}$ in dog and rabbit cardiomycytes. However, direct measurement of $[Ca^{2+}]_{SR}$ have not been achieved in freshly isolated mouse cardiomyocytes.

Here, we show a new protocol optimized to measure $[Ca^{2+}]_{SR}$ in mouse cardiomyocytes. We used electrophysiological and confocal imaging techniques to simultaneously acquire cytosolic (high-affinity Ca^{2+} indicator rhod-2) and SR (fluo-5N AM) Ca^{2+} signals in patch-clamped cardiomyocytes during Ca^{2+} currents and SR Ca^{2+} release. Dialysis via patch-clamp electrode, and reversible permeabilization with streptolysin-O, were sometimes applied in combination to remove contaminations of fluo-5N entrapped in the cytosol. We also recorded spontaneous cytosolic and intra-SR Ca^{2+} waves in permeabilized mouse cardiomyocytes.

The application of this protocol to measure $[Ca^{2+}]_{SR}$ in mouse myocytes will find applications in various experimental studies, for example in mouse models of disease or in transgenic mice exhibiting mutants of RyR or other Ca^{2+} signaling proteins. The technique can help to understand how these diseases and mutations affect $[Ca^{2+}]_{SR}$. Since RyRs are regulated by cytosolic and luminal Ca^{2+} and mutations in RYRs could modify the sensibility to Ca^{2+}, this new protocol will be useful to study how changes in the $[Ca^{2+}]_{SR}$ regulate the RyR activity. Supported by SNF.

3127-Pos Board B282

Identification of Novel Hydroxyl-Benzoquinones as Redox Switchable Calcium Chelators and Potent Biological Antioxidants

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Quinones have important functions in many vital biological processes. We have previously reported novel hydroxylated-quinone structures with a high Ca^{2+}-binding affinity and a putatively high antioxidant capability. However, the molecular mechanisms of the chemical reactions and the physiological significance were not resolved. Here, we analyze the physiologically relevant 2,6-dimethoxy-1,4-benzoquinone (BQ), which is found in wheat and thus part of a everyday diet, but also a precursor in coenzyme Q biosynthesis. We show that BQ can be transformed into novel hydroxyl-benzoquinone forms (OHBQ) and unmask the molecular nature of the chemical reactions. Importantly, the novel OHBQ forms are very stable under physiological conditions and scavenge superoxide radicals formed by primary human monocytes with very high efficiency. Its antioxidant and Ca^{2+}-binding properties can be switched in a redox-dependent manner. The insights into the molecular mechanism of the quinone transformation should increase their usage as powerful antioxidants and facilitate their pharmacological potential.

Membrane Receptors & Signal Transduction II

3128-Pos Board B283

Clusters of Arp2/3 Activators Mimic Pathogenic Actin Comets and Pedestals

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We investigate RTK signaling to the actin cytoskeleton through the Nck adaptor protein. The SH2 domain of Nck binds phosphorylated tyrosine residues and the SH3 domains of Nck bind and activate N-WASP. Activation of N-WASP involves release of the VCA domain. Simultaneous binding of G-actin monomers and actin nucleation factor Arp2/3 to the VCA domain initiates nucleation of a new actin branch. Pathogenic microorganisms induce this pathway to exploit actin polymerization machinery of the host.

Experimental aggregation of a fusion protein containing the Nck SH3 domains on the plasma membrane results in the formation of dynamic actin comet tails. The VCA domain of N-WASP is directly responsible for Arp2/3 activation, and aggregation of VCA can bypass the need for adaptor proteins such as Nck to initiate actin polymerization. We used image analysis to characterize the differences between Nck- and VCA-induced actin structures. Morphometric analysis demonstrates that aggregation of VCA domains on the membrane produced thicker, denser and less elongated actin structures. Particle tracking showed that motile Nck comets move faster than VCA-induced actin structures. Our data indicates that Nck comets reproduce the behavior of Vaccinia comets and VCA clusters resemble EPEC/EHEC actin pedestals. As a consequence of the experimental design, VCA membrane clusters have higher VCA density

than Nck clusters. We therefore co-aggregated trans-membrane VCA domains with ''dummy'' fusion proteins. However, morphology and dynamics of VCA actin structures did not alter significantly, suggesting density alterations cannot explain differences between Nck and VCA-induced structures. We are now testing whether higher VCA turnover and additional Nck binding proteins could explain the more dynamic nature of Nck-induced structures. Understanding the basis for the differences between the Nck and VCA induced actin assemblies will advance our knowledge of RTK signaling to mobilize actin cytoskeleton.

3129-Pos Board B284

Tie2 Receptor Dimerization Mediated by its Extracellular FNIII Domains

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The Tie family of receptor tyrosine kinases (RTKs) regulate a number of angiogenic processes that are critical in vascular development, as well as vascularization of tumor masses. The current signaling paradigm is that angiopoietin (Ang)1 binds to Tie2, promoting Tie2 homodimerization and autophosphorylation. This in turn results in cell proliferation, vessel branching, and sprouting. The extracellular regions of the Tie receptors contain three each of immunoglobulin-like (Ig) domains, EGF-like domains, and fibronectin type III (FNIII) domains. The structure of the Ig/EGF domain region of Tie2 in complex with an Ang protein has been described. However, this structure does not fully explain receptor activation or dimerization. Focusing on the three membrane-proximal FNIII domains - missing from the previously reported structure - we have found that this region can independently drive Tie2 dimerization, indicating that the FNIII domains play an important role in defining the activated dimeric configuration of this receptor. To determine the molecular basis for this observation we have solved a 2.5 Å resolution crystal structure of the Tie2 FNIII domains, which reveals a domain architecture with intermolecular interactions between the second and third FNIII domains that are highly reminiscent of those seen in ligand-induced dimers of the hGHR receptor. Guided by this crystal structure we have generated mutations in the region that defines receptor dimerization. These mutations appear to reduce dimer formation in solution, and to reduce Ang1 stimulated phosphorylation of Tie2 in a cellular context.

3130-Pos Board B285

Studying Protein-Protein Interactions of Receptor Tyrosine Kinases on μ-Patterned Surfaces

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Receptor tyrosine kinases (RTK)s are high-affinity cell surface receptors known to have a critical role in the development of many types of cancer. So far, approximately 20 different RTK classes have been identified. Among them the RTK class I (EGF receptor) and class I (Insulin receptor).

We used an assay combining TIRF microscopy and micro-patterned surfaces, which can be applied for the detection of protein-protein interactions in and near the cell membrane in vivo.

The first part of our work focuses on the Insulin-receptor (IR) and the Insulin-like growth factor 1 (IGF-1) receptor. Like other RTKs, the IR mediates its activity by causing the addition of phosphate groups to intracellular substrate proteins. Thus, cytosolic proteins named Insulin receptor substrates (IRS) are phosphorylated, which finally leads to an uptake of glucose by glucose transporters. We used the μ-patterning assay to analyze the interaction properties of different IRS-proteins with the IR and the IGF1-R. Our results indicate prominent differences in the interaction strength of IRS1 and IRS2 to the IR/IGF1-R, compared to the one of IRS3. FRAP-experiments proved different off-rates of IRS1 and IRS2. In the second part of the presented work we describe the interaction of the epidermal growth factor receptor (EGFR) with an important intracellular binding protein termed Grb2 using the same technique. Performed experiments confirm the strong interaction of these two molecules. Induction with EGF promotes the translocation of Grb2 into Clathrin Coated Pits (CCP)s only within EGFR-enriched membrane regions.

Taken together presented results approve the power of the μ-patterning technique to study the interaction properties of plasma-membrane localized receptors. In the near future our established cellular systems will be used to study the effects of active pharmaceutical ingredients including plant metabolites on the interactions of membrane receptors.

3131-Pos Board B286

Signal Transduction by a Cytokine Receptor: Multi-Scale Computational Studies of the Membrane Associated Gp130 Receptor Complex

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Signal transduction is involved in the control of various essential biological processes such as cell growth, regeneration and apoptosis. Cytokines function as regulators of acute phase response during injury of infection, but are also involved in haematopoiesis, liver and neuronal regeneration. Two gp130 cytokine receptor forms a hexameric complex with two alpha-receptors (IL-6Rα) and two interleukin 6 (IL-6). This receptor complex formation initiates signal transduction via the JAK/ STAT pathway, i.e. tyrosine kinases of the Janus family activate signal transducers and activators of transcription. Some structural information is known about the ectodomain of the gp130 receptor complex, while the structures of the transmembrane and juxtamembrane regions are currently unknown.

Here we apply multi-scale molecular dynamics simulations to shed light on some of the steps occurring within or in proximity to the membrane during signal transduction via the gp130 receptor complex. Coarse-grained (CG) simulations are performed to capture events occurring on long time-scales such as formations of protein-protein and protein-lipid interactions. The information obtained from the CG simulations is then further refined by conversion to atomistic (AT) simulations. The computational studies highlight the effect of lipid composition on protein-protein assembly within the lipid bilayer. Additionally the consequences of the juxtamembrane regions on protein-protein and protein-lipid interactions patterns have been explored and the results reveal a central role of intracellular basic residues not only on the interactions between proteins but also with the membrane. The protein-protein association has additionally been probed in physiological relevant membranes, which are complex in composition and asymmetric between the upper and lower leaflet.

3132-Pos Board B287

Examining the Role of Glycolipids in Integrin and B Cell Receptor Activation

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Glycolipids are an important component of the plasma membrane, and often play important roles in the spatial organization of membrane proteins. Our group is interested in the role of enzymes which alter the glycolipid composition of the plasma membrane in lymphocytes. Glycosyl hydrolase enzymes which catabolize membrane glycolipids have been proposed to regulate receptor signaling by altering membrane glycolipid composition. The human neuraminidase 3 (NEU3) is a membrane-associated enzyme that cleaves terminal neuraminic acid (also known as sialic acid) residues from membrane glycolipids, such as GM3. Cells treated with recombinant NEU3 show changes in their glycolipid composition, thus making NEU3 a powerful tool for probing the role of gangliosides in receptor biophysics. Using single dye tracking (SDT) of membrane receptors by total-internal reflection fluorescence (TIRF) microscopy, we have examined the influence of glycolipids on the lateral diffusion of membrane proteins on lymphocytes. We first examined the T cell integrin, LFA-1. SDT data show clear changes in LFA-1 diffusion, with an increased population of receptors with a large diffusion coefficient after NEU3 treatment. Imaging of LFA-1 by TIRF shows that NEU3 treatment results in co-localization of GM1 and LFA-1, which is distinct from its localization in cells activated with phorbol-12-myristate-13-acetate. To understand the generality of this observation, we examined the influence of NEU3 treatment on the B cell receptor (BCR) complex. NEU3 treatment again increased the proportion of mobile receptors in the B cell membrane relative to control. A similar increase in mobile receptors was observed when cells were activated with PMA. We will present our analysis of SDT and imaging data which support that altering the membrane composition of lymphocytes by NEU3 treatment results in changes to the lateral mobility and subcellular localization of specific receptors.

3133-Pos Board B288

Assessing Metal Ion Preference for the Metal Ion-Dependent Adhesion Site (MIDAS) of Platelet Integrin αIIbβ3

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